International Journal of Pharmaceutics 536 (2018) 63–72

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Sustained-release multiparticulates for oral delivery of a novel peptidic ghrelin agonist: Formulation design and *in vitro* characterization

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ARTICLE INFO

Keywords:
Pellet
Film coating
Sustained-release
Bioactive peptide
Ghrelin

ABSTRACT

There is an impetus to provide appropriate sustained release oral delivery vehicles to protect biofunctional peptide loads from gastric degradation *in vivo*. This study describes the generation of a high load capacity pellet formulation for sustained release of a freely water-soluble dairy-derived hydrolysate, FHI-2571. The activity of this novel peptidic ghrelin receptor agonist is reported using *in vitro* calcium mobilization assays. Conventional extrusion spheronization was then used to prepare peptide-loaded pellets which were subsequently coated with ethylcellulose (EC) film coats using a fluid bed coating system in bottom spray (Wurster) mode. Aqueous-based EC coating dispersions produced mechanically brittle coats which fractured due to osmotic pressure build-up within pellets in simulated media. In contrast, an ethanolic-based EC coating solution provided robust, near zero-order release in both USP Type 1 and Type 4 dissolution studies. Interestingly, the functionality of aqueous-based EC film coats was restored by first layering pellets with a methacrylic acid copolymer (MA) subcoat, thereby hindering pellet core swelling in acidic media. Broadband Acoustic Resonance Dissolution Spectroscopy (BARDS) was utilised as a complementary technique to confirm the results seen in USP dissolution studies. Retention of activity of the ghrelinergic peptide hydrolysate in the final encapsulated product was confirmed as being greater than 80%. The described pellet formulation is amenable to oral dosing in small animal studies in order to assess *in vivo* efficacy of the whey-derived ghrelinergic hydrolysate. In more general terms, it is also suitable as a delivery vehicle for peptide-based bioactives to special population groups *e.g* paediatric and geriatric.

1. Introduction

Oral peptide delivery remains a bottle-neck in the transition of potentially effective therapeutics from bench to bedside (Brayden and Alonso, 2016). Bioavailability of peptides is consistently poor due to the acidic and enzyme-mediated degradation in gut lumen, leading to loss of efficacy. The rapid degradation of bioactive peptide structures *in vivo* necessitates drug delivery technologies which protect the payload in the gastric compartment and allow for site specific delivery to the small and large intestine (Malik et al., 2007). Various formulation approaches have been adopted to protect peptides from degradation within the gastrointestinal tract and increase oral bioavailability, ranging from standard formulations containing functional excipients, to micro- and nano- based (colloidal) delivery systems (Lakkireddy et al., 2016). However, commercial success in terms of an orally active peptide formulation has been limited to a few niche, high potency peptides which can achieve therapeutic efficacy with limited bioavailability (*i.e.* < 1%) (Aguirre et al., 2016). Micro- and nano-based delivery systems encompass a large proportion of the efforts to translate peptide functionality *in vitro* to the clinical setting. However, various limitations exist to these respective approaches: the former typically involves complex processing steps leading to peptide degradation (Witschi and Doelker, 1998; Yin et al., 2008), while the latter displays poor loading capacity (1–5%), variable release characteristics and limited scalability (Jain et al., 2008; Redhead et al., 2001). Furthermore, stresses during processing, including shear forces, exposure to organic solvents and

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https://doi.org/10.1016/j.ijpharm.2017.11.051
Received 25 August 2017; Received in revised form 29 October 2017; Accepted 22 November 2017
Available online 23 November 2017
0378-5173/

excessive drying time will adversely impact on peptide stability, as well as interactions with hydrophilic/hydrophobic interfaces. There is thus an impetus to develop more suitable oral delivery platforms to enable assessment of *in vivo* efficacy for peptidic compounds showing promise in the *in vitro* setting.

The main aim of this study is the encapsulation of a novel bioactive peptide using a traditional multiparticulate formulation approach. These coated pellets are intended for use pre-clinically to investigate bioactive functionality in rodents. In addition, from a clinical utility perspective, pelletised dosage forms offer numerous advantages such as flexible dosing and ease of administration in special population groups. Conventional formulations such as coated pellets are widely used in the pharmaceutical industry to obtain suitable release profiles for a variety of active pharmaceutical ingredients (API) (Lecomte et al., 2004; McGinity and Felton, 2008) . Millimetre size-range pellets have notable advantages compared to sub-micron and colloidal delivery approaches. A narrower particle size distribution allows for homogeneous film formation and more reproducible release profiles. Higher peptide loading can typically be achieved by inclusion of a pelletisation aid such as microcrystalline cellulose. The process is readily scalable to industrial size, while critically this represents a flexible dosing platform ranging from pre-clinical proof of concept studies, to clinical dosing in special population groups, *i.e.* paediatric and geriatric patients. Fluid bed coating technology holds many advantages for coating peptide-loaded matrix pellets. This is a well-established process that allows for simple and efficient polymer layer deposition and subsequent reliable delayed/sustained drug release, depending on the nature of the functional polymeric coat applied. Furthermore, the processing conditions are mild relative to other methods such as pan coating, while low weight gains achieve reliable, uniform coating. Typically, pellets are fluidized by high flow air, while an atomized coating solution or suspension is pulsed onto the pellets. As liquid coating material is deposited and simultaneously dried, the latent heat of evaporation of solvent means that the microenvironment of each individual pellet is considerably lower than the pre-heated inlet air (El Mafadi et al., 2005; Poncelet et al., 2009).

The therapeutic potential of bioactive peptides for treating many health problems, including appetite-related disorders, is becoming increasingly apparent (Torres-Fuentes et al., 2015). Recent work in rodents has demonstrated the ability of whey protein isolate to reduce the expression of satiating genes in the hypothalamus, thereby increasing energy intake (Nilaweera et al., 2017). This study describes a novel peptidic dairy hydrolysate, FHI-2571, with ghrelin receptor agonist activity. Ghrelin, a 28-amino acid containing peptide, is produced in the stomach and functions as an endogenous appetite-stimulant (Howick et al., 2017; Kojima et al., 1999; Nakazato et al., 2001). The ghrelin receptor has thus been a pharmacological target to reduce appetite in obesity as well as to stimulate food intake in conditions of malnutrition and cachexia (wasting syndrome) (Howick et al., 2017; Schellekens et al., 2010). While the precise site of action of ghrelin is still open to some debate (Howick et al., 2017), the high prevalence of the ghrelin receptor throughout the small and large intestinal mucosa is thought to facilitate communication with appetite centres in the brain via the vagus nerve (Date, 2012), and thus may hold potential as a local therapeutic target (Lakkireddy et al., 2016).

Overall, this study aims to first assess the *in vitro* efficacy of a novel ghrelin receptor agonist, FHI-2571, and investigate a formulation approach to progress this bioactive to *in vivo* studies. To overcome the acidic and proteolytic degradation of this whey-derived hydrolysate in the stomach and upper intestine, we have developed a sustained-release oral delivery system to minimise exposure to gastric acid and intestinal peptidases. *In vitro* release profiles of FHI-2571 in traditional USP dissolution tests, confirmed using BARDS, demonstrate the capability of our formulation approach in achieving prolonged, elevated levels of bioactive throughout the small intestine *in vivo*. Activity assays confirm that the peptide retains good bioactive functionality post-encapsulation.

2. Materials and methods

2.1. Materials

Dairy-derived peptide hydrolysate (FHI-2571) was provided by Food for Health Ireland (see Section 2.2). Methacrylic acid and ethyl acrylate copolymer type C (MA, Acryl-EZE® II) and ethylcellulose (EC) (Ethocel™ Standard 20 Premium) were both purchased from Colorcon Corp., Dartford, Kent, UK, while aqueous pseudo-latex of EC (Surelease® Type B NF) was sourced from Colorcon Corp., Indianapolis, IN, USA. Microcrystalline cellulose (MCC, Avicel® PH-101 NF Ph. Eur.) was purchased from FMC Corp., Little Island, Cork, Ireland. Pharmaceutical grade ethanol 96% (v/v) was procured from Carbon Chemicals Group Ltd., Ringaskiddy, Cork, Ireland. Unless otherwise stated, only deionised water was used in this study. For the Ca^{2+} mobilisation assays, fetal bovine serum (3.3%) was obtained from Sigma-Aldrich, Arklow, Wicklow, F7524. Assay buffer was composed of 1 × Hanks balanced salt solution, HBSS, Gibco™ 14,065,049 (Thermo Fisher Scientific™), containing 20 mM HEPES (Sigma-Aldrich, Arklow, Wicklow, H0887). The endogenous agonist, ghrelin (rat), was supplied by Tocris Bioscience, Avonmouth, Bristol, UK (Cat. No. 1465).

2.2. Generation of FHI-2571

A dairy-peptide hydrolysate was prepared by a method similar to a previously published method (Mukhopadhya et al., 2015). Briefly, bovine milk derived whey protein (80% w/w protein, Carberry Group, Ballineen, Cork, Ireland) was suspended at 10% protein (w/w) in reverse osmosis-treated water and agitated continuously at 50 °C for 1 h in a jacketed tank. The pH was adjusted using a NaOH 4.0 N solution (VWR, Dublin, Ireland). A bacterial food-grade enzyme preparation, was added to the protein solution until 7–12% degree of hydrolysis was achieved. The enzyme was then inactivated by heat-treatment and the resultant hydrolysate solution was dried in a Niro TFD 20 Tall-Form Dryer (GEA, Düsseldorf, Germany).

2.3. Ca^{2+} Mobilisation assay for peptide ghrelin receptor activity pre- and post-encapsulation

Ghrelin receptor mediated changes in intracellular Ca^{2+} mobilisation were monitored on a FLIPR Tetra High-Throughput Cellular Screening System (Molecular Devices Corporation, Sunnyvale, California, USA). Ca^{2+} mobilisation assays were performed according to the manufacturer's instructions and modified from a previously described method (Pastor-Cavada et al., 2016). Human Embryonic Kidney (HEK293A) cells stably transfected with the ghrelin receptor were seeded in black 96-well microtiter plates at a density of 3×10^4 cells/well and maintained overnight at 37 °C in a humidified atmosphere containing 5% CO_2. Twenty-four hours before the experiment, media was replaced with serum-free media containing 1% non-essential amino acids (NEAA). On experimental day cells were incubated with 80 µL of 1 × Ca5 dye in assay buffer (1 × Hanks balanced salt solution – HBSS, supplemented with 20 mM HEPES buffer) according to the manufacturer's protocol (R8141, Molecular Devices Corporation, Sunnyvale, CA). Addition of compound (40 µL/well) was performed by the FLIPR Tetra, and fluorescent readings were taken for 120 s at excitation wavelength of 485 nm and emission wavelength of 525 nm. The relative increase in cytosolic Ca^{2+} was calculated as the difference between maximum and baseline fluorescence and depicted as percentage relative fluorescent units (RFU) normalized to maximum response (100% signal) obtained with 3.3% fetal bovine serum (FBS). Background fluorescence was recorded in cells in assay buffer alone and subtracted from RFUs. All compounds and hydrolysates used in experiments were prepared in assay buffer. FBS (3.3%) and the endogenous agonist

ghrelin (1465; Tocris) were used as positive controls of Ca^{2+} influx. Responses were considered as positive when Ca^{2+} influx exceeds 20% compared to control. Data were analysed using GraphPad Prism software (PRISM 5.0; GraphPAD Software Inc., San Diego, California, USA). Sigmoidal concentration-response curves were generated using non-linear regression analysis with variable slope.

2.4. Pellet preparation by extrusion-spheronisation

Initial process optimisation studies, focusing on pellet production, trialled a range of different ratios of FHI-2571 to MCC (50:50, initially). The gradual reduction of FHI-2571 content from 50% afforded a robust pellet with minimal generation of fines at 33% peptide loading (data not shown). Therefore, requisite quantities of FHI-2571 and MCC were combined in a ratio of 33:67, respectively, and manually blended for 1 min. The powder was then added to a Kenwood Major planetary mixer (KM005, Kenwood Ltd., Hampshire, UK), fitted with a 'K' blade mixing arm, and dry blended at minimum setting for 5 min. The resultant dry powder blend was granulated by addition of deionised H_2O, under constant agitation by planetary mixer at minimum setting. A homogenous dispersion of moisture was ensured, by adding deionized H_2O periodically from an atomizer every 15 s. Mixing was stopped every 2–3 min, to scrape material down from the sides of the mixing vessel. The granulation end-point was achieved upon addition of a cumulative amount of deionisedH_2O equivalent to 45% (w/w) of the dry powder blend. The granulate was immediately extruded at an extrusion speed of 17–19 rpm using a sieve extruder (Caleva® Extruder 20, Caleva Process Solutions, Sturminster Newton, Dorset, UK). Both the screen thickness and aperture diameter were 1 mm. The extrudate was subsequently spheronized for 1.5 min at a speed of 1500 rpm, using a Caleva® Spheroniser 250 (Caleva Process Solutions, Sturminster Newton, Dorset, UK) equipped with a cross-hatch friction plate having a diameter of 22.5 cm. Resulting pellets (c. 100 g) were dried in a laboratory scale microfluid bed system (Vector Corp., Marion, IA, USA) at 40 °C for 20 min and then stored at room temperature in an airtight container until further testing took place.

2.5. Pellet film coating

Film coating was performed in a laboratory scalemicrofluid bed system, equipped with a Wurster funnel insert, in bottom-spray mode. Both nozzle air (16.6–16.7 psig) and airflow (310–335 L/min) were adjusted to maximum setting. Liquid feed rate (gram/minute) and spray pattern parameters varied, depending on the film coating polymer mixture (see Table 1). Various coating polymer mixtures were prepared; a concentrated 25% (w/w) aqueous pseudo-latex of EC (Surelease® Type B) was diluted to 11% (w/w), using deionised H_2O water. Dissolution of EC (Ethocel™ Standard 20 Premium) in 96% (v/v) EtOH to produce a 5% (w/w) ethanolic EC polymer coating solution was performed slowly under conditions of vigorous agitation. Dry methacrylate powder (Acryl-EZE® II) was gradually dispersed in deionised H_2O water to produce a 10% (w/w) coating mixture. All of the coating polymer mixtures were subjected to constant agitation at 900 rpm, for not less than 30 min, using a magnetic stirrer at room temperature. Agitation of the polymer coating mixtures (750–850 rpm at room temperature) was maintained during film coating procedures. Uncoated pellets were charged to the coating vessel (25–50 g, pellet load), and the coater reassembled. Pellets were pre-heated in-situ, for approximately 10 min (inlet air temperature 80 °C; outlet air temperature ~ 50 °C), prior to commencing film coating. The amount of coating polymer required for film coating was based on a pre-determined weight gain, based on dry uncoated pellet mass.

The duration of the film coating process was determined by the theoretical percentage of coating required, and the dry weight of pellets added to the spray coater. The vessel containing the coating polymer mixture was weighed before and after the coating process, to determine the actual weight of coating solution sprayed onto the pellets. The microfluid bed coating system was visually monitored to ensure that a steady uniform flow of pellets was maintained within the spray chamber.

Table 1
Coating parameters. Coating parameters employed during the film coating with methacrylic acid and ethyl acrylate copolymer type C (Acryl-EZE® II), aqueous dispersion of ethylcellulose (Surelease® Type B), and organic solution of ethylcellulose (Ethocel™ Standard 20 Premium) polymer mixtures, respectively.

Film Coating Parameter		10% (w/w)Acry-LEZE® II in water	11% (w/w) Surelease® Type B in water	5% (w/w) Ethocel™in 96% (v/v) EtOH
Liquid Feed Rate (g/minute)		0.8	1.0	Minimum
Spray Pattern	On (minute)	0.4	0.4	Continuous
	Off (minute)	0.1	0.1	

2.6. pH susceptibility tests

Powdered FHI-2571 (6 g) was dissolved in 100 mL of deionised H_2O and aliquoted into 4 × 25 mL samples. Next, 3 M HCl was added to bring the individual solutions to the requisite pH (pH 1, 3, 5 and untreated), using a pHenomenal® 1000 L pH meter with a pHenomenal® 221 pH electrode. After pH was adjusted, samples were incubated for 30 min under gentle agitation. Finally, 50 µL of each sample was removed and added to 950 µL of Ca^{2+} assay buffer and neutralization of acidic pH confirmed before samples were added to cells.

2.7. In vitro dissolution tests

2.7.1. USP type 1 (Basket) dissolution studies

Dissolution testing (USP Type 1) was performed, using a basket-type dissolution apparatus (DISTEK, Inc., Model 2100C, North Brunswick, NJ, USA) with 500 mL of both simulated gastric fluid sine pepsin (SGFsp) (pH 1.2) and simulated intestinal fluid sine pancreatin (SIFsp) (pH 6.8) as dissolution media. Dissolution bath temperature was maintained at 37 ± 0.5 °C. Impeller shaft speed was 50 rpm. Dissolution medium sampling was conducted at predefined timepoints (10, 20, 40, 60, 90, 120, 180, 240, 300, and 360 min) from a location not less than 1 cm from the vessel wall and midway between the top of the rotating impeller and dissolution media surface. After sampling, an equal volume of dissolution medium was added to the dissolution vessel.

2.7.2. USP type 4 (Flow-through) dissolution studies

An Erweka® flow-through apparatus (Model DFZ 720, ERWEKA GmbH, Germany), equipped with a HKP 720 piston pump and 22.6 mm diameter cells, was used to perform USP type 4 dissolution studies. The temperature of the water bath was maintained at 37 °C. Experiments were carried out over six hours using the closed loop system at a flow rate of 4 ml/min. The dissolution media was composed of 100 mL SGFsp for the first two hours. SGFsp was then replaced with 100 mL of SIFsp, after two hours. Samples (1 mL) were taken at the same time intervals as for USP Type 1 dissolution (described above). After sampling, an equal volume of dissolution medium was added to the dissolution vessel.

2.8. Peptide quantification assay

The bicinchoninic acid (BCA) assay was performed using a BCA assay kit (Thermo Fisher Scientific™ Pierce™ BCA Protein Assay, Catalog Number 23225) according to a previously published method.

Diluted stock samples were made using a 2 mg/ml stock solution of FHI-2571 in SGFsp. Using this stock solution, a serial dilution was performed to afford six 0.1 mL solutions with concentrations of 2, 1, 0.5, 0.25, 0.125, and 0.0625 mg/mL, respectively. This method was repeated, using SIFsp. A 25 µL volume of each sample obtained during dissolution testing (see Section 2.6) was then transferred to a 96-well plate. After the dissolution experiment was completed, peptide not released from the formulation was determined. Remaining pellets were removed, physically crushed and redissolved in the relevant media. The quantity of liberated peptide was determined using the BCA assay. Working reagent was prepared by mixing BCA assay Reagent A with BCA assay Reagent B in a ratio of 50:1. The working reagent (200 µL) was then transferred to each well. The plates were then covered and incubated at 37 °C for 30 min. Spectrophotometric analysis (Flexstation II Multiplate Fluorometer, Molecular Devices, Sunnyvale, California) was performed at 562 nm. A standard curve was made by plotting the average blank-corrected absorbance (562 nm) for each BCA assay standard *versus* concentration (mg/mL). The standard curve was used to determine the protein concentration of each unknown sample.

2.9. Electron microscopy

Samples were mounted onto aluminium stubs using double sided carbon tape. All samples were sputter coated with a 5 nm layer of gold palladium (80:20) using a Quorum Q150 RES Sputter Coating System (Quorum Technologies, UK), before being examined using a JEOL JSM 5510 Scanning Electron Microscope (JEOL Ltd., Japan) in the BioSciences Imaging Centre, Department of Anatomy & Neuroscience, UCC. Digital electron micrographs were obtained of areas of interest.

2.10. Broadband acoustic resonance dissolution spectroscopy

A Broadband Acoustics Resonance Dissolution Spectroscopy (BARDS), as described previously by Fitzpatrick et al. (2014) was used to investigate the BARDS responses (BARDS ACOUSTIC SCIENCE LABS, Cork, Ireland). A sample size of 0.1 g was used in each experiment. Testing was performed under acidic conditions, using 25 mL simulated gastric fluid (SGFsp pH 1.2) as solvent. The stirrer rate was set to 300 rpm. Prior to testing, temperature (c. 25.5 °C), relative humidity (c. 47%), and pressure (c. 1025 mbar) were recorded. Before sample addition, steady state resonances were recorded for 30 s, while the magnetic follower was in motion. Spectra were recorded using a microphone (Sony ECM-CS10, range 100 Hz–16 kHz) for 560–1300 s. The frequency time course of the fundamental frequency curve is shown as manually extracted data from the recorded acoustic response. All experiments were performed in triplicate ($n = 3$) and average values with error bars representing the standard deviation are presented.

2.11. Data analysis

Data were analysed and graphs generated using both GraphPad Prism software and Microsoft Excel software. All means were calculated from the results of at least three independent experiments carried out in triplicate. For the *in vitro* calcium mobilization assays, standard error of the mean (SEM) is depicted, while all dissolution results report standard deviation (SD).

3. Results

3.1. A dairy-derived peptide exhibits ghrelin receptor agonist activity in vitro

The activity of the whey-derived hydrolysate, FHI-2571, on the ghrelin receptor was shown using intracellular Ca^{2+} mobilization, as a measure of downstream ghrelin receptor signalling activation (Schellekens et al., 2013), in HEK293A cells (human embryonic kidney cells) stably expressing the ghrelin receptor tagged with an enhanced green fluorescent protein (GHSR-1a-EGFP) (Fig. 1A). FHI-2571 hydrolysate stimulated calcium mobilization in cells expressing ghrelin receptor in a concentration-dependent manner, with the EC_{50} = 1.1mg/ml and efficacy (E_{max}) reaching 205%. The potency of FHI-2571 is 1000-fold lower compared to the endogenous receptor ligand, ghrelin (EC_{50} = 2.84 µg/ml). Interestingly, the maximal response attained for FHI-2571 hydrolysate is higher compared to ghrelin (E_{max} = 150%). Efficacy of both compounds was normalised to the maximal response of the positive control (3.3% FBS, E_{max} = 100%). Critically, the concentration response curve shows that the FHI-2571 hydrolysate has ghrelin receptor agonist activity, while the un-hydrolysed parent whey protein concentrate (WPC) fails to elicit appreciable activity in the same assay (Fig. 1A). Furthermore, no Ca^{2+} influx was observed in wild-type HEK293A cells (HEK293A-WT) not expressing the ghrelin receptor, when exposed to the FHI-2571 hydrolysate, while treatment with control (FBS) resulted in a non-specific maximal intracellular Ca^{2+} mobilization in this cell line (Fig. 1B), indicating the specificity of FHI-2571 activity on the ghrelin receptor.

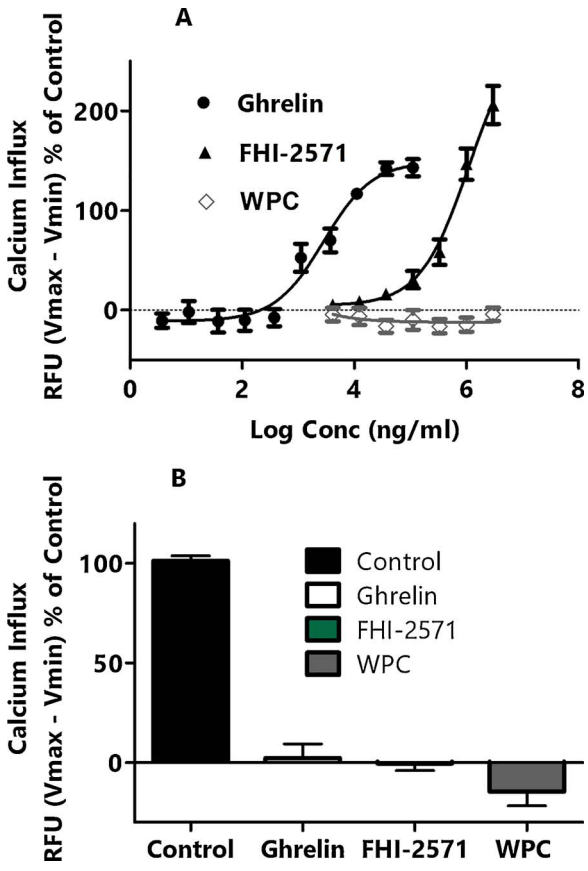

Fig. 1. (A) Concentration response curve of novel whey-derived hydrolysate. Concentration response curves for the endogenous ghrelin receptor ligand, ghrelin, the whey-derived hydrolysate, FHI-2571 and the parent whey protein concentrate (WPC) measured in ghrelin receptor over-expressing HEK293A cells. Intracellular Ca^{2+} increase was depicted as a percentage of maximal Ca^{2+} influx in relative fluorescence unit (RFU) as elicited by control (3.3% FBS). Graph represents mean ± SEM of three independent experiments performed in triplicate. Fig. 1 **(B): No activity of test compounds on wild-type cells.** Activity of control (FBS), ghrelin, FHI-2571 and parent whey protein concentrate (WPC) in wild-type (HEK293A-WT) cells (representative of three independent experiments carried out in triplicate).

3.2. pH susceptibility of FHI-2571

The FHI-2571 hydrolysate was exposed to varying degrees of acidic pH for a time representative of minimum gastric residence time in the fasted state (minimum 30 min (Tuleu et al., 1999)). A pH dependent

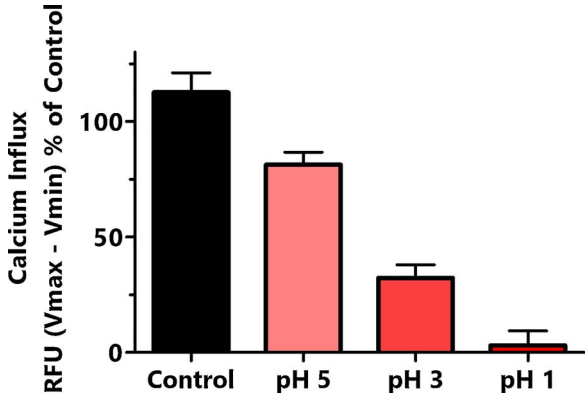

Fig. 2. Ghrelin receptor agonist, FHI-2571 displays pH dependent activity.
Graph represents three independent experiments carried out in at least triplicate. Reduction in FHI-2571-mediated ghrelin receptor activation following exposure to acidic pH confirms the need for an oral delivery mechanism (Control = FHI-2571 not exposed to acidic pH, RFU = Relative Fluorescence Units).

loss in peptide activity is observed for the whey-derived FHI-2571 (Fig. 2), confirming the requirement for a protective film coat to minimise exposure to gastric acid before progression to *in vivo* efficacy studies.

3.3. Aqueous-based ethylcellulose dispersion yields a mechanically weak film coating

USP Type 1 (Basket) dissolution studies were carried out in both simulated gastric conditions (simulated gastric fluid, SGFsp, pH 1.2) in order to assess the release profile of peptide from the pellets. Pellets displayed burst release of the peptide, with > 80% release over the first 60 min. This occurred independent of coating thickness, as 10% coating represented no additional benefit to the 5% (Fig. 3). Visual investigation showed film disintegration or “shelling” occurred within 20 min of

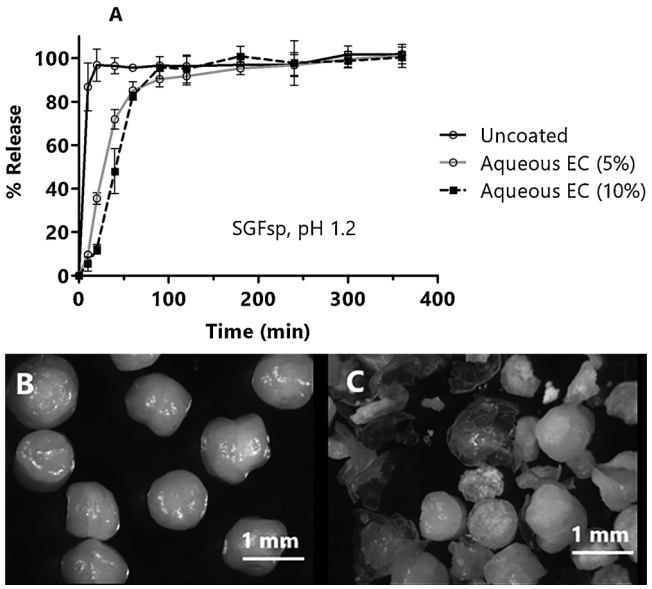

Fig. 3. Dissolution study of aqueous-based Ethylcellulose and FHI-2571 hydrolysate pellets.
USP Type 1 (Basket) dissolution studies (50 rpm, 37.5 °C) showed a burst release in simulated conditions with > 80% release over the first hour in both uncoated and coated FHI-2571 hydrolysate pellets following exposure to Simulated Gastric Fluid sine pepsin (SGFsp) pH 1.2 (A). Graph represents three independent experiments carried out in triplicate. Macroscopic investigation showed an unexpected disintegration or “shelling” of the coat from the pellets, resulting in rapid release of peptide (before introduction of media, B, and after, C).

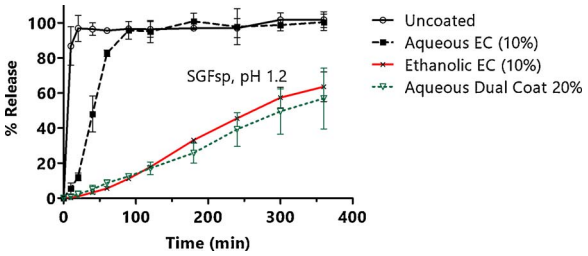

Fig. 4. Dissolution study of FHI-2571 hydrolysate pellets with ethanolic-based ethylcellulose film coat and aqueous-based dual coat.
Two different approaches were taken to circumvent the phenomenon of "shelling" as a result of coating with an aqueous dispersion of EC. (1) An organic solution of EC was applied to create a more robust coat, and (2) a methacrylic acid copolymer was layered beneath the aqueous EC to form a functional acid-resistant subcoat. USP Type 1 (Basket) dissolution studies (simulated gastric fluid, SGFsp, pH 1.2) showed effective delayed release for both organic EC and aqueous dual-coated pellets. Graph represents three independent experiments carried out in triplicate.

exposure to the aqueous medium. This was attributed to the relatively low mechanical strength of the film coat produced from aqueous dispersion-based fluidized coating (Lecomte et al., 2004; Siepmann et al., 2008). An aqueous-based dispersion is hence an unsuitable coating approach to achieve sustained delivery of whey-derived FHI-2571 hydrolysate.

3.4. Towards pellet coating achieving sustained release of peptide – Ethanolic solutions of ethylcellulose vs. aqueous dispersion based dual-coat

Two alternative strategies were employed to circumvent the observed film disintegration for EC coats prepared using aqueous-based EC dispersions (Fig. 4). Firstly, an ethanolic solution of EC was prepared and applied to the pellets. The EC coated pellets prepared from ethanolic solutions achieved near-zero order delayed release in simulated USP Type 1 (Basket) dissolution studies carried out in SGFsp. Due to the drawbacks associated with organic solvent use (Muschert et al., 2009; Srivastava and Mishra, 2010), an aqueous-based dual coat approach was trialled as an alternative. A pH-resistant methacrylic acid copolymer subcoat was applied to the pellets in order to prevent water ingress to the pellet core under acid conditions. Interestingly, this aqueous-based dual coat approach achieved a similar delayed release profile as the organic EC coat.

3.5. Scanning electron microscopy investigation of whole and cross-sectioned pellets

Fig. 5 represents SEM images of uncoated, aqueous EC coated, ethanolic EC coated and dual-coated pellets (A–D, respectively). Notably, images reveal no obvious structural cracks or pores on the surface of the coated pellets which may explain the fluid ingress and film coat rupture in the aqueous-based EC coated pellets. Fig. 6 presents the SEM images obtained from cross-sectioned pellets with the various film coats. The aqueous EC coated pellet displays a porous cross-sectioned coat (Fig. 6B). The porous nature of this coat can also be seen in the outer layer of the dual coated pellets (Fig. 6F). By contrast, the ethanolic-based EC coat is distinctly non-porous and waxy in appearance (Fig. 6D) while the methacrylate-based subcoat is also visibly non-porous (Fig. 6F). The porous nature of the aqueous-based EC coat is likely responsible for the osmotic-induced fluid ingress to the pellet core, and subsequent film disintegration. Both the ethanolic based EC coat and the methacrylate based subcoat are functionally resistant to water ingress in simulated gastric conditions. This is attributable to the non-porous substructure evident in the photomicrographs.

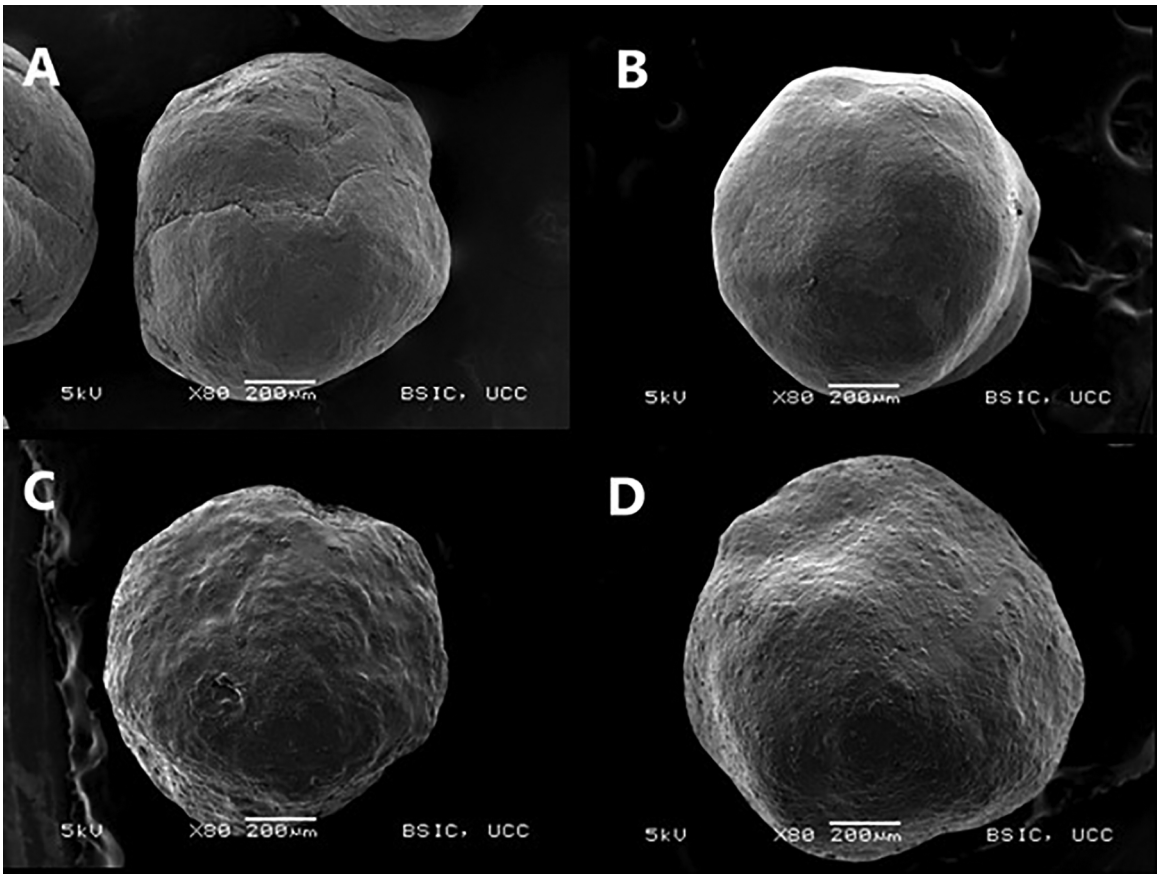

Fig. 5. Scanning Electron Microscope (SEM) images of whole pellets.
SEM images of uncoated (A), aqueous ethylcellulose (EC) coated (B), ethanolic EC coated (C) and dual-coated pellets (D).

3.6. Investigation of peptide release in pH transfer model

A pH transfer model was utilised in order to investigate whether there was any appreciable difference in release when intestinal pH was introduced to the pellets after 2 h exposure to SGFsp (pH 1.2) conditions (Fig. 7). Predictably the aqueous EC-coated pellets display a burst release of peptide with > 80% release in the first hour. Notably, the dual-coated EC pellets retain a sustained release profile after the transition from acid pH, to intestinal pH (pH 6.8). In contrast to the release profiles observed under USP Type 1 conditions, the ethanolic-based EC coat also displays a faster release rate compared to the dual-coat. This difference in release patterns between Type 1 and Type 4 may be attributed to different flow patterns and agitation between the systems.

3.7. Broadband acoustics resonance dissolution spectroscopy (BARDS)

As expected in the case of uncoated pellets, a distinct change in real-time compressibility of the solvent is evident immediately after addition of the pellet to the solvent with the fundamental curve approaching its respective frequency minimum within 170 s (Fig. 8). This reflects the rapid disintegration/dissolution of the pellets lacking a protective film coat. In the case of both the dual-coated pellets and organic-based EC-coated pellets, there is no noticeable dissolution/disintegration event, following addition to the acidic solvent in the vessel (SGFsp pH 1.2). This spectral observation was confirmed by visual inspection; both the dual-coated pellets and organic-based EC-coated pellets remained intact during testing and no disintegration was observed. In the case of the aqueous-based EC coated pellets, a distinct change in solvent compressibility was observed from 400 s, indicating that the coated pellets undergo a dissolution/disintegration event here under acidic conditions. This was also evident visually in the solvent vessel with pellets undergoing disintegration. The aqueous-based EC coated pellets demonstrated a lag time, prior to disintegration, where the frequency minimum is observed at 780 s. This result indicates that the aqueous-based EC pellets do not remain intact in an acidic environment and further support findings from previous dissolution experiments (see Figs. 3A,).

3.8. Ghrelin receptor activity post-encapsulation

In order to quantify the impact of processing conditions on bioactivity of the peptide cargos, activity of the encapsulated peptide *in vitro* was reassessed. Activity of the FHI-2571 hydrolysate liberated from the encapsulated product was determined relative to activity of non-encapsulated FHI-2571 peptide in the ghrelin receptor overexpressing cells, as before. Activity was quantified as being greater than 80% for organic EC coated and dual coated pellets (Fig. 9).

4. Discussion

Dairy-derived peptides are increasingly recognised for their bioactive components which may bestow clinical benefits (Hartmann and Meisel, 2007; Torres-Fuentes et al., 2015). Peptides fractions have been isolated with ACE-inhibitory action, and blood-pressure lowering properties of these dairy-derived bioactives *in vivo* have been reported. Furthermore, a casein-derived bioactive fraction with specific serotonin-2C receptor (5-HT_{2C}) agonist activity eliciting satiating properties in a rodent model has been described (Schellekens et al., 2014). Ghrelin and the growth hormone secretagogue receptor (GHSR-1a) or ghrelin receptor, play an important role in energy balance and appetite regulation (Howick et al., 2017; Schellekens et al., 2010). Many studies have reported potent appetite-stimulating effects of both peripheral and

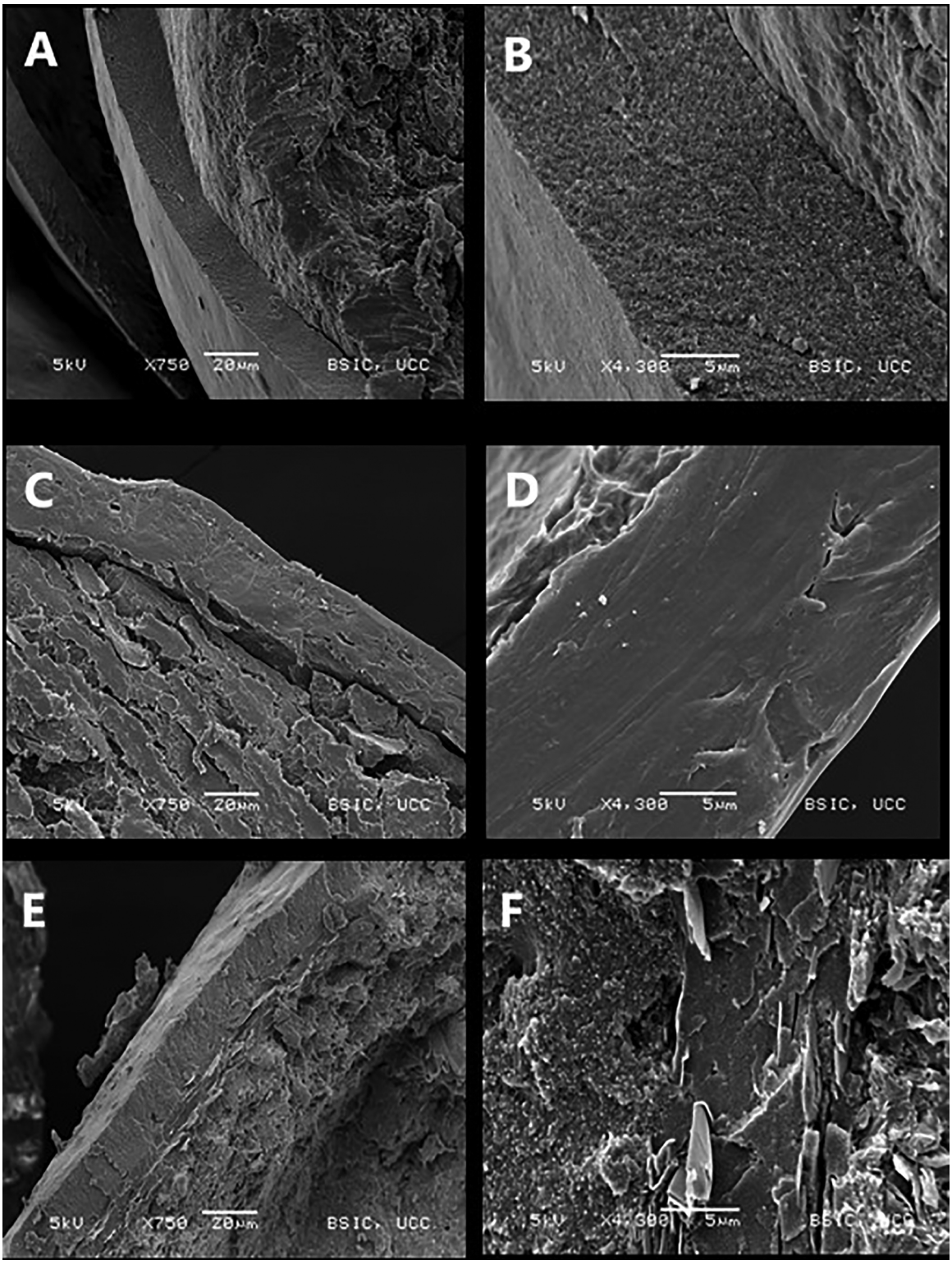

Fig. 6. Scanning Electron microscope (SEM) images of cross-sectioned pellets.
Aqueous EC coated pellets have a distinctly porous coat which allows for water ingress and increased osmotic pressure within the pellet, leading to swelling and rupture of the film coat (A,B). Organic EC coated pellets display a more complete, non-porous coat (C,D), while the aqueous dual coated pellets show a distinct double layer, with the porous EC overcoat and an impervious, acid resistant subcoat (delineated with white arrows) (E,F).

central administration of ghrelin (Tschop et al., 2000; Wren et al., 2001). In this study, we identified a milk whey-derived hydrolysate with intrinsic ghrelin receptor agonist activity. The dairy hydrolysate, FHI-2571, dose-dependently and specifically increased intracellular Ca^{2+} in HEK293A cells heterologously expressing the ghrelin receptor *in vitro*, while the unfractionated parent whey elicited negligible effects on the receptor (Fig. 1A). *In vivo*, the ghrelin receptor is present throughout the small and large intestine, acting remotely via the vagus nerve to communicate with appetite centres in the brain (Date, 2012; Date et al., 2000). Given the appropriate oral delivery mechanism, a

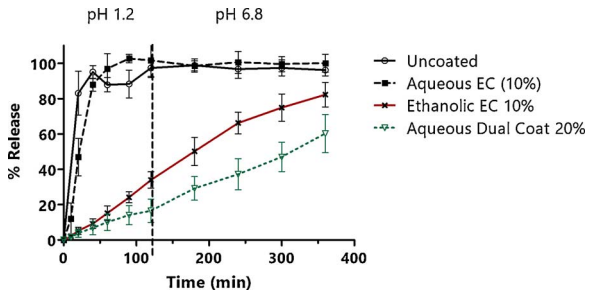

Fig. 7. Modelling of release profiles in a pH transition setup.
USP Type IV (Flow-through) dissolution experiments were carried out using 22.6 mm diameter cells to quantify FHI-2571 release in a pH-transfer model (pH 1.2–pH 6.8). A closed loop system maintained at 37 °C recirculated 100 mL of media at 4 ml/min for the duration of the experiment (adapted from a previous study (Keohane et al., 2016)). This yielded a predictable sustained release for both organic EC coated and dual-coated pellets. Graph represents three independent experiments carried out in triplicate. Dashed line indicates transition from SGFsp (pH 1.2) to SIFsp (pH 6.8) at 120 min.

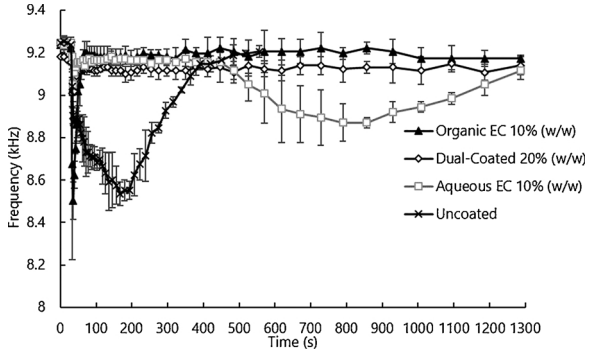

Fig. 8. Fundamental curve frequency of coated pellets in SGF.
Fundamental curve frequency time course of uncoated (Δt = 167 s), aqueous-based EC coated (10% w/w) (Δt = 790 s), dual-coated (MaA and aqueous EC, 10% w/w, respectively), and organic-based EC (10% w/w) pellets, containing FHI-2571, in 25 mL SGFsp (pH 1.2). This data is representative of three independent experiments carried out, and demonstrates the comparable integrity of the dual-coated pellets with that of the organic-based EC pellets, in low pH conditions. Δt denotes the time at which minimal frequency reached (release) is reached.

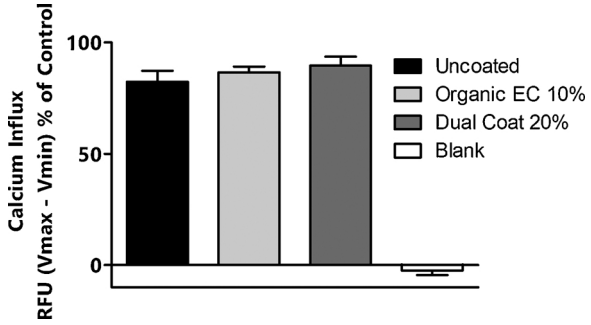

Fig. 9. FHI-2571 retains bioactive functionality after encapsulation.
Activity of FHI-2571 after encapsulation was determined relative to activity of non-encapsulated FHI-2571 on ghrelin receptor overexpressing HEK293A cell line (representative of four independent experiments carried out in at least triplicate). Activity was quantified as being greater than 80% for organic EC and dual coated pellets.

potential to increase food intake *in vivo* by targeting intestinal ghrelin receptor therefore exists.

Given the acidic and peptidase rich environment of the gastrointestinal tract, as well as the barriers to epithelial absorption, development of appropriate delivery platforms to improve *in vivo* efficacy of bioactive peptides is required (Brayden and Alonso, 2016; Gleeson et al., 2016). Microspheres and microcapsules are one such approach, however degradation of peptide due to complex processing steps is a concern (Witschi and Doelker, 1998; Yin et al., 2008). Furthermore, there is an ever-increasing interest in nano-sized formulations (Date et al., 2016). Nano-based approaches offer a platform to traverse membrane barriers and deliver peptide drugs in a targeted manner, increasing oral bioavailability and favourably altering pharmacokinetic profiles (Griffin et al., 2016). However, much work remains to be done in order to elucidate the mechanisms of action and safety profiles of nano-formulations. Critically, despite the exciting advances in the micro- and nano-fields, none are yet proven as a viable, industrially scalable delivery approach to achieve both high loading of peptide, and a predictable release pattern. On the other hand, there are limited examples of conventional mm-sized pellets being used to deliver peptide payloads, despite the approach being widely used in formulation of small organic drug molecules. This is traditionally due to the poor permeation of peptides across the intestinal barrier, extensive first-pass metabolism and short half-life in the body, not to mention the high concentration of peptidases present in the upper small intestine. In the case of the bioactive peptide under investigation here, FHI-2571, its pharmacological target, the ghrelin receptor, is found throughout the small and large intestine on vagal afferent terminals located just beyond the mucosal brush border (Date et al., 2000), while a substantial proportion of the hydrolysate size fraction is < 1 kDa, meaning that paracellular transit to these nerve terminals is possible (Griffin and O'Driscoll, 2011). The ghrelin receptor is also located in the myenteric plexus of rodent and human gastrointestinal tract (Takeshita et al., 2006), furthering the case for enhancing the delivery of the peptidic payload to the intestinal lumen. Therefore, we sought to develop a simple, high loading sustained release delivery vehicle to protect the ghrelinergic peptide from acid exposure in the gastric compartment and upper small intestinal breakdown, to facilitate *in vivo* proof-of-concept studies.

Firstly, the need for a gastro-protected delivery vehicle was validated by exposing FHI-2571 to acidic pH, which predictably abolished the bioactivity of this compound on the ghrelin receptor in a progressive manner (Fig. 2). Polymeric film coating of active pharmaceutical ingredient (API)-loaded core pellets has been widely utilised, with predominantly aqueous based functional polymer coatings (Siepmann and Siepmann, 2013). EC is the most commonly-used coating polymer. It is non-toxic and biodegradable, and achieves predictable, pH independent release profiles due to drug diffusion across a water-insoluble membrane (Ozturk et al., 1990). Generally, aqueous EC colloidal dispersions are preferred as a coating medium due to safety and environmental reasons (Muschert et al., 2009; Srivastava and Mishra, 2010). Moreover, it is possible to achieve higher percentages of solid content in aqueous dispersions; the high viscosity of organic solutions of EC is a limiting step for coating media (Lecomte et al., 2004). Therefore, coating time can be excessively lengthened when organic solutions are used.

In our study, aqueous-based EC dispersion failed to provide us with a sufficiently robust film coating during release testing. Burst release of peptide was observed (> 80% in the first 60 min) in USP Type-1 and USP-Type 4 apparatus. Macroscopic and microscopic investigation showed that the polymer coating fractured after exposure to aqueous media, allowing peptide to freely-diffuse out of the matrix system though fluid-filled cracks, rather than diffusing through the polymeric coat. This was attributed to the mechanism of film coat formation for a coating dispersion. During the fluid bed coating process, evaporation of solvent on the surface of the particles leads to sequential, layered polymer chain packing. These discrete polymer particles interact with one another via relatively weak Van der Waals interactions. Aqueous solubility is a major factor affecting osmotic pressure within coated pellets in contact with dissolution media. Osmotic pressure is a driving force for water ingress into pellets, increasing the intra-particulate volume and outward pressure on the coating film. Furthermore, migration of API into the film coat during the fluid-bed coating process has also been reported (Melegari et al., 2016). Therefore, the high aqueous

solubility of the peptide may lead to leaching into the EC coat, creating water soluble pores which affords easier ingress of water into the pellet core, thereby causing swelling and an increased intra-particulate pressure. Considering the high loading of peptide in our system (33%), this problem is compounded leading to pellet swelling and film fracture.

Here, we demonstrate the mechanical integrity of two alternative film coating approaches, which both provide time-dependent release of a bioactive peptide in the *in vitro* setting. This is particularly useful in the context of sustained delivery of hydrophilic peptides. Initial burst release has been reported from some reservoir devices, which tapers over time due to a reducing concentration in the reservoir (Dekyndt et al., 2015). Organic solutions of the film coating polymer lead to greater mechanical strength in the resulting coat. This is due to the fluid movement of the polymer chains in solution, which, upon removal of the solvent phase, cross-link and form a robust, physically-bonded polymeric meshwork (Lecomte et al., 2004). This is supported by SEM images (Fig. 6) which show a distinct porous nature to the cross sectionedaqueous-based film coat (Fig. 6B), compared to a more complete, non-permeable structure seen in the ethanolic-based film coat (Fig. 6D). Consistent with the impervious nature of the coat show in SEM cross-sections, FHI-2571 -loaded pellets coated with an ethanolic solution of EC displayed near-zero order release in both USP Type 1 (Basket) and USP Type 4 (Flow-through) dissolution setups (Figs. 4 & 7).

Due to the drawbacks associated with organic-based coating solutions, an aqueous –based coating approach to achieving an appropriate release profile of active peptide was desirable. Increased efficiency of aqueous EC film coats has been demonstrated by allowing a curing step to take place post-encapsulation, which typically involves extended periods of exposing the product to high temperature and humidity – water is an efficient plasticizer for many polymers (Kucera et al., 2013; Siepmann and Siepmann, 2013). However, in this study an extended period of exposure to such harsh conditions was not possible due to the probability of peptide hydrolysis. Furthermore, layered multi-particulates have been used successfully by Siepmann and colleagues to provide reliable zero-order release of water-soluble agents (Dekyndt et al., 2015). However, this involved incorporating the drug into the film coating layer itself. The potential to incorporate a peptide-based bioactive into such coating solutions, rather than the pellet matrix itself, is limited due to high risk of denaturation.

Dual coated pellets have been used before to increase the functionality of the outer coat and optimise release profiles. In this study, an acid resistant methacrylic acid (MA) co-polymer was proposed, which was layered beneath the aqueous EC coat in order to provide an impermeable seal-coat in acid conditions. This may be considered atypical, given that a pH dependent polymer would normally form the outer layer in dual-coated systems. The acid-resistant layer was initially trialled as an overcoat of the aqueous EC coating, which limited burst release in acid conditions. However, upon transition to intestinal pH conditions, immediate dissolution of the MA overcoat occurred, with subsequent “shelling” of the EC subcoat (data not shown). This was due to the swelling of the pellet core combined with the mechanically brittle subcoat formed by the aqueous-based EC dispersion. USP-Type 1 dissolution studies for our dual-coated pellets in SGFsp display a near-zero order release profile comparable to organic EC pellets (Fig. 4). This is consistent with the insolubility of methacrylic acid below pH 5.5, which likely prevented osmotic fluid ingress into the pellet core, and subsequent pellet swelling and film fracture. Furthermore, USP-Type 4 dissolution studies, also show a delayed release profile after transitioning to intestinal media (pH 6.8) (Fig. 7). This may be considered surprising given the solubility of the methacrylate copolymer above pH ~ 5.5. The intact EC overcoat in this case is likely hindering the access of the intestinal buffer to the surface of the subcoat, thereby reducing the rate at which the subcoat can dissolve. The advantage to the MA applied as a subcoat is therefore two-fold, initially it prevents the ingress of fluid to the pellet core and subsequent pressure-induced film fracture. Secondly, the limited exposure of intestinal media to the MA subcoat due to the intact EC overcoat serves to slow the overall dissolution of the film coat.

BARDS was utilised to confirm the release profiles obtained from the compendial dissolution methods (Fig. 8). This is an emerging technology used to explore the changes in compressibility of a solvent that occurs during dissolution. During an experiment, the introduction of the pellets into the BARDS system causes changes in the speed of sound in the dissolution medium, which can be monitored acoustically. The dissolution process thus generates a change in the resonance frequency time course of the solvent in the vessel. BARDS analysis has previously shown successful application in the analysis of powder blend uniformity (Fitzpatrick et al., 2012) and the profiling of enteric-coated drug delivery systems (Fitzpatrick et al., 2014).

Of vital importance to this work was to confirm that the active peptide retains its bioactivity post-encapsulation, as protein aggregation or denaturation may occur during formulation. In fluid-bed coating, inlet air temperature is partially negated due to the latent heat of evaporation of the coating polymer solvent during the spraying process (El Mafadi et al., 2005; Poncelet et al., 2009). This leads to a milder micro-temperature at the surface of individual pellets than would be suggested by the process parameters themselves. In our study, peptide liberated from the pellet formulation displays > 80% activity of the untreated peptide. Compared to alternative methods of encapsulation which have been used for peptides incorporating solvent-based methods, we consider this to be a reasonable retention of activity in light of our processing conditions.

In conclusion, a multiparticulate sustained release formulation approach for delivery of a ghrelin agonist peptide is described. Aqueous-based EC film coats applied to pellets in the millimetre size range are porous and mechanically brittle, leading to disintegration or “shelling” of the coat in aqueous media. Here, we observed that the high loading of a freely soluble ghrelin agonist peptide enhanced the problem of film disintegration due to increased osmotic pressure and pellet swelling. To overcome this, we provide near zero-order release by taking two alternative approaches: (1) Organic EC based solution can be applied to the pellets, or (2) an aqueous dispersion of a pH dependent MA co-polymer may be introduced as a subcoat to the aqueous EC. This provides an impermeable seal coat in gastric conditions which prevents fluid ingress into pellets, thereby preventing pressure-induced EC layer fracture and allowing the EC polymer to function as originally intended. Both processes allow the ghrelinergic peptide to retain sufficient activity after encapsulation. In conclusion, we designed a successful delivery formulation for a peptide based ghrelinergic dairy-derived bioactive hydrolysate. This delivery platform is suitable for progression to pre-clinical rodent models to assess efficacy *in vivo*.

Declaration of interest

The authors declare no conflict of interest.

Acknowledgements

The above described work was funded by Enterprise Ireland under grant number TC20130001. The authors gratefully acknowledge the help and expertise provided by Dr. Ken Devine (School of Pharmacy, UCC), Dr. Eoin Murphy, Dr. Phil Kelly and Dr. Kate Barry (Teagasc Food Research Centre, Moorepark, Fermoy, Co. Cork). The authors would like to acknowledge Suzanne Crotty and the Department of Anatomy & Neuroscience Imaging Centre, BioSciences Institute, University College Cork, for assistance in preparing and imaging specimens for this research. The authors would also like to thank Prof. Melissa Hanna-Brown and Dr. Abina Crean for access to the Pfizer BARDS Instrument.

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